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MAINTENANCE OF NEURONAL IDENTITY AND FUNCTION

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Maintenance of neuronal identity and function

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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ABSTRACT

Neurons in the CNS acquire specific characteristics throughout development and maintain them for the entire lifespan. Instructive regulation of the transcription machinery ensures the acquisition of identity by spatiotemporal patterns of transcription factor expression. Additionally, gene silencing of alternative lineages is important for specification of neuronal identity and function, however the mechanisms regulating transcriptional repression are poorly understood.

In this study we focused on epigenetic regulation of gene expression and how perturbation of it can lead to loss of function and disease.

In **Paper I** we characterized histone modifications on two clinically relevant neuronal populations, the dopaminergic and serotonergic neurons implicated in Parkinson's disease and depression respectively. We studied the repressive modifications H3K27me3, H3K9me3 and active H3K4me3 and associated them with gene expression levels throughout cell maturation, from NPCs to adult postmitotic neurons. The generated comprehensive map also illustrates how drug induced stress in dopaminergic neurons alters the histone modifications pattern affecting gene expression.

After acquisition of identity, **Paper II** focuses on how this can be maintained throughout life. Removal of H3K27me3 in dopaminergic and serotonergic neurons resulted to erroneous gene expression patterns and progressive loss of neuronal function. In dopaminergic neurons electrophysiological and molecular properties were perturbed in a region specific manner, with SNc being the most vulnerable, phenomenon similar to PD development. Mice showed phenotypic impairments with motor symptoms or anxiety-like behavior in cell-type dependent manner.

In **Paper III** DNA methylation in neuroblastoma tumors was examined as a potential therapeutic approach. Hypermethylated loci keep potential tumor suppressors silenced leading to cancer formation. Here we showed that combined administration of the demethylating AZA with RA that promotes neuronal differentiation exhibited a favorable outcome in tumor growth. Further analysis of treated xenografted tumors revealed EPAS1 as a potential tumor suppressor, opposite to previously published data assigning the gene as an oncogene.

LIST OF SCIENTIFIC PAPERS

I. A comprehensive map coupling histone modifications with gene regulation in adult dopaminergic and serotonergic neurons.
Erik Södersten, **Konstantinos Toskas,** Vilma Rraklii, Katarina Tiklova, Åsa K Björklund, Markus Ringnér, Thomas Perlmann, Johan Holmberg
Nature Communications, 2018 Mar 26;9(1):1226

II. PRC2-mediated repression is essential to maintain identity and function of differentiated dopaminergic and serotonergic neurons.
Konstantinos Toskas, Behzad Yaghmaeian-Salmani, Olga Skiteva, Wojciech Paslawski, Linda Gillberg, Vasiliki Skara, Irene Antoniou, Erik Södersten, Per Svenningsson, Karima Chergui, Markus Ringnér, Thomas Perlmann, Johan Holmberg
(*Manuscript*)

III. Combined epigenetic and differentiation-based treatment inhibits neuroblastomatumor growth and links HIF2α to tumor suppression.
Isabelle Westerlund, Yao Shi, **Konstantinos Toskas**, Stuart M.Fell, Shuijie Li, Olga Surova, Erik Södersten, Per Kogner, Ulrika Nyman, Susanne Schlisio, Johan Holmberg
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LIST OF ABBREVIATIONS

5-HT	Serotonin
6 OHDA	6 Hydroxydopamine
Aadc	Aromatic amino acid decarboxylase
AZA	5-Aza-deoxycytidine
ChIP	Chromatin Immunoprecipitation
DA	Dopamine
DEGs	Differentially expressed genes
EED	Embryonic Ectoderm Development
ESCs	Embryonic Stem Cells
EZH2	Enhancer of Zeste homolog 2
FACS	Fluorescence activated cell sorting
GO	Gene ontology
H3K27me1	Histone 3 Lysine 27 monomethylation
H3K27me2	Histone 3 Lysine 27 dimethylation
H3K27me3	Histone 3 Lysine 27 trimethylation
H3K4me3	Histone 3 Lysine 4 trimethylation
H3K9me3	Histone 3 Lysine 9 trimethylation
HIF2α	Hypoxia Inducible factor 2 alpha
HP1	Heterochromatin protein 1
Jmjd3	Jumonji domain-containing protein D3
NB	Neuroblastoma
NPC	Neuronal progenitors
OSN	Oct4 Sox2 Nanog
PcG	Polycomb group
PFC	Prefrontal Cortex
PRC2	Polycomb repressive complex 2
PD	Parkinson's disease
RA	Retinoic acid
RbpAp 46/48	Retinoblastoma suppressor associated protein 46/48
SERT	Serotonin transporter

SNc	Substantia Nigra pars compacta
SUZ12	Supressor of Zeste 12
TF	Transcription factor
TH	Tyrosine Hydroxylase
TPH2	Tryptophan Hydroxylase 2
TrxG	Trithorax
VMAT2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area

1 INTRODUCTION

1.1 INTRODUCTION TO CHROMATIN AND GENE EXPRESSION

Multicellular organisms have a remarkable characteristic of creating distinct cell types from an essentially invariable genome. This wide variety of gene programs is achieved due to cellular signaling events that regulate the activity of cell-type specific DNA-binding transcription factors acting as key regulators of gene expression [1]. However, additional regulation of gene expression through processes affecting chromatin structure and histone modification have been identified [2]. One prominent example of this is the repression associated with Polycomb group (PcG) genes that initially were discovered in *Drosophila melanogaster* as maintenance factors required for proper Hox gene expression and body segmentation [3]. PcG act antagonistically with Trithorax (TrxG) group components and regulate a wide variety of cellular processes, including X chromosome inactivation, genomic imprinting, cell cycle control, as well as playing a role in stem cell biology and cancer[4].

1.1.1 Nucleosomal Structure

The main component of chromatin is the nucleosome, consisted of 146bp of DNA, wrapped around double copies of four core histones, H2A, H2B, H3 and H4. The N-terminals of histones can be modified with acetylation, methylation, phosphorylation, ubiquitination and sumoylation. Acetylation creates a structural relaxation to the chromatin by adding negative charge, whereas all the other modifications seem to act as a docking site for effector protein complexes to be recruited and regulate gene expression. Histone modifications that are related with gene activation, such as trimethylation of Histone H3 at lysine 4 (H3K4me3), promote the recruitment of protein complexes with ATP-dependent remodeling activity[5]. As a result, nucleosomes become more mobile, facilitating the relaxation of chromatin and accessibility of DNA so that the transcription machinery can initiate gene expression. On the contrary, histone modifications that are involved in gene repression, such as trimethylation of histone H3 at lysine 9 (H3K9me3) and at lysine 27 (H3K27me3), act as docking sites for repressor complexes with methyltransferase activity [6,7]

1.1.2 Role of PRC2 in cell fate regulation

PRC2 is the only known protein complex with methyltransferase activity toward H3K27 and is in charge for all the H3K27 methylation in mouse embryonic stem cells (mESCs)[8] . The

different types of methylation (H3K27me1, me2, me3) are found in different genomic distributions. H3K27me1 is found on gene bodies of actively transcribed genes. H3K27me2 is very abundant, found in 50%-70% of all H3 in a cell and located in intra- or intergenic regions preventing false binding of enhancers and promoters [8-10]. H3K27me3 is mostly detected in regions overlapping with PRC2 components and is considered the hallmark of PRC2 mediated transcriptional repression.

Its catalytic component of the complex resides in the EZH2 subunit, which when on its own stays inactive. On the contrary, catalytic activity and in vivo stability is achieved only when binding with two other PRC2 subunits, EED and SUZ12 takes place. The fourth component of PRC2, RbAp46/48, is also necessary for H3K27 methyltransferase activity[11-14]. PRC2 holds a high affinity binding with its own catalytic product, H3K27me3 through the EED protein. EED protein can recognize and bind to H3K27me3 creating a positive feedback loop, since this interaction stimulates the catalytic activity of the complex[7,15]. That could also be a potential mechanism through which H3K27me3 is spread throughout the genome and ensures the propagation of the histone mark on newly synthesized chromatin[15]

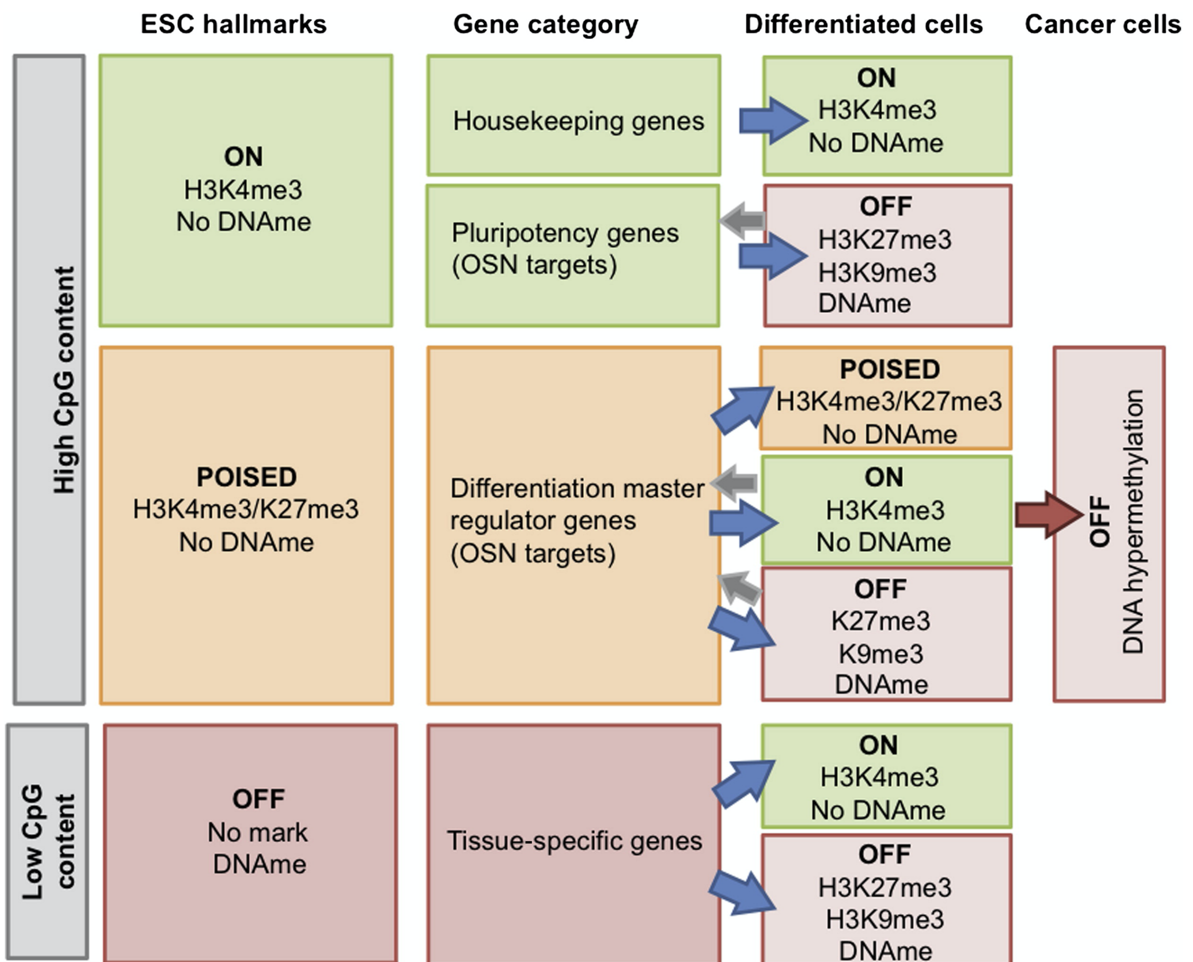
PcG mutants displayed developmental abnormalities in a range of species. Knockout of *Ezh2, Eed or Suz12* leads to embryonic lethality in mouse embryos [14,16]. Tissue specific knockouts of these genes have revealed important roles of PRC2 in coordinating cell fate transitions in the development of tissues. Loss of PRC2 impairs the stability of cell identity and impedes cell differentiation, thus alterations in PRC2 activity can lead to uncontrolled proliferation of dividing cells causing adverse outcomes including cancer

1.2 TOWARDS ACQUISITION OF NEURONAL IDENTITY

The chromatin structure in pluripotent embryonic stem cells (ESCs) has different features compared to the structure in differentiated cells. In ESCs structural proteins have hyperdynamic interactions with chromatin[17] , and the total transcriptional activity is higher than the one in postmitotic cells [18] . This phenomenon could be explained by the high levels of chromatin-remodeling factors that maintain the accessibility of the open chromatin status. It has been reported that loss of self-renewal capacity in ESCs has been observed upon depletion of remodeling proteins Chd1 or BRg1 [18,19].

ESCs are characterized from their ability to differentiate into different cell types. Genes that are critically involved in the differentiation process, when they remain silent, they have a permissive chromatin that is occupied from both H3K27me3 and H3K4me3 around the

transcriptional start sites. This bivalency plays a critical role by maintaining the differentiation genes silent in the ESCs, while it keeps them poised for activation upon specific developmental pathways [20,21].



Adapted from Barrero et.al 2010, Cell Stem Cell

Fig.1: Epigenetic cell fate mapping

These bivalent domains are also characterized by lack of DNA methylation [22,23] and concomitantly by the presence of poised RNA polymerase II at the transcription initiation site[24]. In that way, important regulatory sequences and master transcription factors remain accessible and responsive during early stages of development.

This highly dynamic equilibrium of histone modifications is regulated by histone modifying enzymes, the PcG proteins adding the H3K27me3 and the TrxG adding the H3K4me3 marks.

However, during differentiation, the chromatin dynamics change and the permissive chromatin including the bivalent genes is no longer accessible for gene expression [25]. Once the cells are committed to a certain lineage, genes that are involved in alternative lineages, as well as pluripotency genes, should be permanently silenced. As a matter of fact, pluripotency

genes lose the active mark H3K4me3 but gain the repressive ones H3K27me3, H3K9e3 and DNA methylation, whereas bivalent genes lose the H3K4me3 but retain the H3K27me3 [26,27]. It has been reported that in fetal lung fibroblasts, repression is enhanced by an additional layer of the repressive histone modification H3K9me3 or DNA methylation, ensuring permanent gene silencing of developmental genes[28] (Fig.1).

During the transition from pluripotency to differentiation the removal of histone marks is actively regulated by demethylases. As such, H3K27 demethylases UTX and Jmjd3 participate in the activation of Hox genes during development [29] and associate with MLL complexes that are depositing the H3K4 marks on these loci. However, it is not clear yet why there are bivalent domains that remain in this status in differentiated adult cells.

Additionally, gene repression during differentiation is monitored by the methyltransferase G9a that is catalyzing the methylation of H3K9 at regulatory regions of pluripotency genes, which eventually leads to binding of heterochromatin protein 1 (HP1) and de novo DNA methylation[30]

1.2.1 Dopaminergic neurons

Dopaminergic (DA) neurons are responsible for releasing the catecholaminergic neurotransmitter, dopamine. They produce tyrosine hydroxylase (TH), the rate limiting enzyme in the synthesis of catecholamines and are found throughout the nervous system, including the ventral midbrain. Midbrain DA (mDA) are organized in three different nuclei : the *substantia nigra pars compacta* (SNc), the ventral tegmental area (VTA) and the retrorubral field (RrF). According to their location, mDA neurons project to distinct areas controlling or modulating specific functions[31]. VTA and RrF DA neurons project to the prefrontal cortex and regulate emotional behavior, natural motivation, reward and cognitive function and are primarily implicated in a range of psychiatric disorders. On the contrary, DA neurons located in the SNc project to the dorsolateral striatum forming the nigrostriatal pathway which mainly regulates motor function and degenerates in Parkinson’s disease (PD).

In rodents, midbrain DA neurogenesis takes place in the ventricular zone of the floorplate (FP) when mDA progenitors divide to generate postmitotic neurons at E10.5[32,33]. Several different transcription factors are critical for the maintenance of differentiation of mDA, notably PITX3 and LMX1B [34-39]. Moreover, Engrailed-1 and 2 (EN1,EN2), neurogenin 2 (NGN2), NURR1 and TGF-β also influence mDA differentiation[40-42]. Importantly,

expression of the dopamine neurotransmitter phenotype is dependent on NURR1 transcription factor which regulates proteins important for the synthesis of TH and DAT as well as receptor related proteins such as vesicular monoamine transporter 2 (VMAT2) and RET receptor tyrosine kinase[40,43-45].

The metabolic pathway in the presynapse includes synthesis, degradation, compartmentalization, release and reuptake. Dopamine is synthesized from the precursor L-DOPA produced from the tyrosine provided from the brain capillaries. Synthesized dopamine is packed into vesicles through vesicular monoamine transporters (VMAT2). These vesicles can be either released into the synaptic cleft or serve as a reserve pool in the presynapse. Once released in the synaptic cleft, part of the released neurotransmitter diffuses in the cleft and part of it is retrieved by dopamine transporter (DAT) proteins and returned back to the presynaptic terminal for recycling. Dopamine is released in the synaptic cleft either by a constant, low rate release or through the activated release induced by action potential stimulus[46]

1.2.2 Serotonergic neurons

A small number of brainstem neurons are specialized to use serotonin (5-HT) as a neurotransmitter. Serotonin acts as a neuromodulator, adjusting neuronal excitability according to the 5-HT receptors engaged [47,48]. It also has long term effects on cell function such as trophic effects on growth [49]. Although the amount of 5-HT produced in the brain is a small percentage of the total body 5-HT synthesis, it has been implicated in a wide range of functions from basic homeostatic processes, like thermoregulation and breathing[50] to more elaborate ones such as mood control[51,52], memory[53] and reward[54], aggressive behavior and maternal care[55,56].

Brain 5-HT neurons are located in the hindbrain, organized mainly in clusters along the midline, called raphe nuclei. From these nuclei, 5-HT axons radiate broadly to innervate the whole central nervous system from the olfactory bulb to the spinal cord[57]. Together with the highly divergent anatomical organization, 5-HT neurons exhibit various molecular and physiological identities as well as distinctive neural circuits, explaining their diverse brain functions[58] .

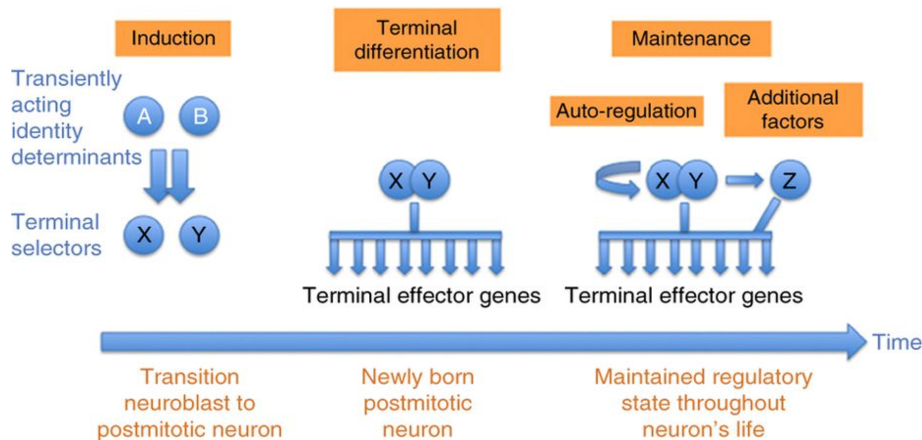
It has been shown that 5-HT neurons are among the earliest generated ones, they are produced at mid-gestation (embryonic day (E) 9.5-12) in rodents. Newborn neurons have to

migrate to their adult raphe locations, develop axonal structures, acquire firing characteristics and make connections with neuronal targets[59]. The maturation of 5-HT neurons in rodents starts with the initiation of 5-HT synthesis and continues to at least the third week of life[60]. The 5-HT molecular identity comprises from a defined transcriptome that encodes stably expressed terminal effector proteins necessary to build and maintain 5-HT connectivity, neurotransmission and synaptic responsivity to environmental stimuli. This transcriptome is not unitary for all the subpopulations but they are all characterized by their capacity to biosynthetically make 5-HT[61].

1.3 MAINTAINING POSTMITOTIC NEURONAL IDENTITY

What defines a specific neuron subtype is not only the anatomical and electrophysiological characteristics they acquire during development but also the well defined gene expression patterns that remain stable throughout their life [62,63]. Neurotransmitter identities and transporters that synthesize, synaptically package and reuptake a certain neurotransmitter remain stable in adult neurons for over decades. However, the mechanism that governs this stability and maintenance of post-mitotic differentiated traits across the lifespan is poorly understood. Our current knowledge of neuronal maintenance has been acquired through studies of serotonergic, dopaminergic and noradrenergic neurotransmitter identity [64-68]. A wide range of studies have shown that removal of individual transcription factors (TFs) after the acquisition of transmitter identity led to decreased expression of markers of transmitter phenotype in the respective neuron type [69,70]. Deletion of Pet-1 or Lmx1b in fetal post-mitotic serotonin (5-HT) neurons resulted to severe reduction of all markers of the 5-Ht neuron type battery [67,70]. In a similar way, targeting Gata-3 in fetal 5-HT neurons showed defects in the maintenance of tryptophan hydroxylase 2 (Tph2), aromatic amino acid decarboxylase (Aadc), serotonin transporter (Sert) and vesicular monoamine transporter 2 (Vmat2) expression [70]. Mesencephalic dopamine (DA) neurons of murine fetuses showed severe and progressive loss of DA identity and DA transmitter in substantia nigra (SN) and ventral tegmental area (VTA) after depletion of the nuclear hormone receptor gene (Nurr1). In early adulthood, targeting Nurr1 resulted to similar outcome with transcriptional downregulation of tyrosine hydroxylase (TH), dopamine transporter (DAT) and VMAT2 as well as reduction in the striatal levels of DA however, the number of mutant cell bodies remained unaffected[65]. Similarly, deletion of Pet-1 or Lmx1b in adult 5-HT neurons results to expression changes in Tph2 ,Sert and VMAT2 showing that sustained expression of key identity genes is highly dependent on adult TFs. However, expression levels of AADC and Htr1a remain unaffected

after adult depletion of Pet-1 indicating that transcription factor requirements might evolve with aging and additional factors could replace their activity[70] .



Adapted from Deneris et.al 2014 Nature Neuroscience

Fig.2: Initiation and Maintenance of neuronal identity

The continuous expression of terminal selector transcription factors can be explained through the autoregulatory concept which holds on the binding of the TF to its own regulatory region serving as a "lock in" mechanism. Additionally, maintaining the expression of terminal selector genes is achieved due to the dedicated maintenance factors, like the engrailed (En) homeodomain paralogs, En1 and En2, factors that are important for the maintenance of dopaminergic and serotonergic identity but not for their acquisition [71,72] (Fig.2). Chromatin modifications have also been implicated in regulating the activity of transcribed genes as shown by the histone H3 methylation at Lysine 4 [73], though not sufficiently since TFs are continuously required for the activity of those genes [74]

1.4 EPIGENETICS IN NEUROBLASTOMA

Mutated forms of PcG and TrxG proteins have been reported in several human tumors [75], suggesting that changes in the respective histone marks affect cancer progression. Additionally, cancer cells are often characterized with aberrant DNA methylation on CpG islands, silencing cancer related genes, including many tumor suppressors.

Neuroblastoma is a pediatric cancer of the sympathetic nervous system. It is the most frequent solid tumor diagnosed in young children and is characterized by heterogenous clinical outcome. It originates from neural crest cells of the sympathoadrenal lineage and can form anywhere in the sympathetic nervous system. Due to low mutation rate found in neuroblastoma patients, research is focusing on epigenetic regulation of tumor formation,

such is DNA methylation[76,77]. Drugs resulting in hypomethylation have already been approved by FDA and EMA for treatment of certain malignancies, therefore interest focuses on how they affect NB tumor progression. 5-aza-2´deoxycytidine (AZA) is an analogue of cytosine, substituting cytosine in the newly synthesized DNA strand and therefore disturbs DNA methylation by DNA methyltransferase (DNMT1)[78] . AZA is used as a mediator of DNA demethylation aiming to activate tumor suppressors that have been transcriptionally silenced. Similarly, HDAC inhibitors have been used synergistically with retinoids resulting to favorable outcome in neuroblastoma tumor growth [79].

The exact mechanism of this phenomenon is not clear yet, however the administration of DNA methyltransferase and histone deacetylase (HDAC) inhibitors as a therapeutic way, supports the relevance of the epigenome in the malignant formation[80]

2 RESEARCH AIMS

The aim of this study is to investigate the mechanism that governs maintenance of neuronal identity throughout lifespan. Apart from the instructive regulation provided by transcription factors controlling cell type specific gene expression, there is presumably a need to permanently silent alternative transcription programs. However, it still remains unclear how permanent repression of aberrant transcription is achieved in mature neurons. A central question is whether, once repression has been established, the stable silencing of alternative gene programs is dependent on continued presence of instructive factors or can be passively sustained through a non-permissive chromatin state characterized by DNA-methylation and repressive histone modifications.

In this direction, we focused on two well studied and highly clinically relevant neuronal populations, raphe nuclei serotonergic neurons and midbrain dopaminergic neurons, perturbations in which are related with depression and Parkinson's disease respectively.

In **Paper I,** we analyzed histone modifications related with active or repressed chromatin state and identified their chromatin targets in dopaminergic and serotonergic neuronal populations.

In **Paper II,** we focused on the role of the PRC2- regulated histone marks for the maintenance of the lifelong cell identity in dopaminergic and serotonergic neurons.

In **Paper III,** we investigated the response and growth of high-risk neuroblastoma tumors after treatment with epigenome targeting drugs.

3 RESULTS

3.1 PAPER I

“A comprehensive map coupling histone modifications with gene regulation in adult dopaminergic and serotonergic neurons.”

Post transcriptional modifications play an important role in cell fate determination during neuronal differentiation and also during the entire lifespan of the neuron. However, technical challenges didn’t allow until that time, characterization of histone modifications further than in vitro cultures where millions of cells were necessary for ChIP experiments. In this study we implemented a method that allowed us to perform ChIP-sequencing experiments in as few as 1000 cells per reaction, enabling us to characterize sparse neuronal populations such are the dopaminergic and serotonergic neurons of the mouse brain. Combined with RNA seq experiments, we associated the repressive (H3K27me3, H3K9me3) or permissive (H3K4me3) histone modifications with gene expression levels in neuronal progenitors (NPCs), midbrain dopaminergic neurons (mDA) or serotonergic neurons (SER).

Isolation of postmitotic neuronal nuclei was performed through FACS based on a fluorescent reporter mCherry that was exclusively expressed in the populations of interest. NPCs were SOX2 positive progenitors, isolated with the use of fluorophore tagged antibody. After validating the purity (96,6%) of the sorted population with single cell sequencing, genes were assigned a chromatin state having exclusively either one of the analyzed histone modifications a combination of them, or none, enabling 8 potential chromatin states of the genes. Chromatin states were associated with different levels of gene expression and correlated well with repressive or permissive chromatin states .

During mDA development, chromatin states were reflecting the transition from NPCs to postmitotic neurons with genes gaining H3K4me3 when expressed in mDA and conversely, gaining H3K27me3 and/or H3K9me3 when they were repressed during differentiation. Interestingly, genes already decorated with broad H3K27me3 in NPCs were downregulated in mDA and gained additional repressive mark of H3K9me3. Moreover, downregulated genes transitioning from K4>K4 showed decreased enrichment of the H3K4me3 mark, contrary to the promoter of upregulated genes. Interestingly, silent developmental genes upon differentiation remained silent and acquired H3K9me3 acting as an additional layer of repressive chromatin state.

In a similar way transition from NPCs to SER neurons was characterized by the deposition of the chromatin marks in a cell type specific manner, highlighting the fact that transcription factors important for neuron subtype specification had a layer of repressive histone modifications as a safeguarding mechanism against erroneous activation. Bivalent genes in NPCs were selectively derepressed during differentiation and de novo bivalent marks occurred in postmitotic neurons in correlation with cell type specific gene expression.

To investigate how chromatin modifications corelate with cell plasticity and environmental stress, we examined the patterns of repressive and permissive histone modifications under drug induced stress. After administration of 6 OHDA in the mouse brain, a neurodegenerative stress induction specifically targeting dopaminergic neurons we observed derepression of genes. Half of those genes were decorated with H3K27me3 and/or H3K9me3, belonging to apoptotic pathways GO terms. More specifically K4/K27 bivalent genes were the most enriched state of upregulated genes.

Similarly, after exposure to the recreational drug methamphetamine, we observed derepression of genes in mDA belonging to bivalent state. In the contrary, bivalent genes in SER neurons did not show any gene expression change.

3.2 PAPER II

"PRC2-mediated repression is essential to maintain identity and function of differentiated dopaminergic and serotonergic neurons"

After acquisition of cell type specific characteristics, neurons need to maintain their identity over the entire lifespan. Transcription machinery must instructively express cell specific genes and at the same time keep the alternative lineages silent. The mechanisms regulating gene silencing are poorly understood though. In this study we aimed to elucidate the role of PRC2 mediated repression in the maintenance of neuronal identity over lifetime. For that reason we utilized the methodology of our previous study on the two clinically relevant neuronal populations, the dopaminergic and serotonergic neurons.

We conditionally deleted *Eed*, one of the core components of PRC2, in *Slc6a3* (DAT) expressing neurons at the embryonic day 13.5, resulting to Eed ablation in dopaminergic neurons at the day of birth (P0). Interestingly, it was only 30 days after birth that there was a significant reduction of H3K27me3 in these neurons as IHC experiments revealed, highlighting the necessity of PRC2 for the long term presence of the histone mark.

The loss of EED in mDA neurons had a progressive effect in the gene expression level of its target genes. In four months old mutant mice, there was a 75% reduction of K27 positive genes, that peaked in 8 months old mice where there was no K27 positive gene. Among the upregulated genes, there were early developmental and pattern specification genes accompanied by increased levels of H3K4me3 mark. Interestingly, key regulating dopaminergic genes were significantly downregulated in the 8 months mice.

Along with the gene expression changes, reduced TH immunoreactivity and dopamine metabolites were observed in the midbrain and striatum of 8 months mutants, without any cell loss as revealed by cell quantification in the midbrain tissue. All the above, would indicate that neuronal function could have been perturbed in these mice, which was validated with altered electrophysiological properties and mouse behavioral deficits.

Additionally, loss of PRC2 in serotonergic neurons had a tremendous effect in the key features of the population. Lack of H3K27me3 resulted to a progressive effect on serotonin specific gene expression and function. Mice at 8 months of age, exhibited downregulation of serotonergic gene battery, reduction in the levels of serotonin in the raphae and PFC as well as anxiety behaviour. Of note, histone modification analysis of differentially expressed genes showed that among the derepressed genes the K9/K27 chromatin state was the most frequently represented in both serotonergic and dopaminergic populations.

Histological analyses of the dopaminergic neurons indicated that the effect of PRC2 ablation was population dependent. We then generated single nuclei RNA seq data from 8 months mDA neurons and their analysis showed that the nuclei originating from the SNc were the ones with most differentially expressed genes. Early developmental genes, like Hoxd were upregulated and the biggest fold decrease in mDA signature genes (*Th, Slc6a3,En1,Nr4a2*) was observed in the SNc cluster.

3.3 PAPER III

“Combined epigenetic and differentiation-based treatment inhibits neuroblastoma tumor growth and links HIF2α to tumor suppression.”

One feature of high risk neuroblastoma is the increased levels of DNA methylation in the promoter regions of potential tumor suppressors. High risk tumors are characterized by MYCN amplification and loss of heterozygosity in the 1p36 locus . Treatment therapies include administration of retinoids for induction of neuronal differentiation. However, many

of the high-risk NBs do not respond to such treatment and alternative approaches need to be adopted. In this study we wanted to understand how a combination of the DNA demethylating agent 5-Aza-deoxycytidine (AZA) with retinoic acid (RA) would impact tumor growth in NBs where RA only was not effective.

In vitro culture of NB cell lines (1p36-) treated with combination of AZA and RA, showed decreased proliferation and induction of neuronal differentiation. The results were validated *in vivo*, as tumor cells were xenografted in immunodeficient nude mice and treated with AZA+RA, resulting to reduction of tumor growth. NB cells were injected at day 0 (D0) and treatment started either at D0 or at D8 showing delayed tumor growth in both cases. Even after termination of treatment (D22), tumor growth was twice slower as without treatment, until they reached the experimental endpoint.

RNA sequencing and methylation analysis of endpoint xenografted tumors showed a significant effect on gene expression, but no correlation between the upregulated genes and the unmethylated promoters. We therefore sought to examine earlier timepoints, at which treatment started having effect. With treatment starting at D8, we harvested the tumors at D14 and analysis of the DEGs revealed hypoxia related genes among the upregulated ones. *EPAS1* (encoding the HIF2α protein) was upregulated at D14, however this effect could have been as a result of tumor growth and hypoxic conditions created within the tumor. In vitro experiments though, revealed that elevated levels of EPAS1 was an actual response of the AZA+RA treatment.

Interestingly, the promoter of EPAS1 showed a significant yet minor demethylation status that could hardly explain the gene upregulation. Additional xenografts with the usage of HIF2a inhibitor, validated the role of EPAS1 as tumor supressor in NB tumors.

In NB tumors from patients, there was a strong correlation between increased levels of EPAS1 and favorable outcome of the patients further supporting our data suggesting that HIF2a may act as a tumor suppressor in NB.

4 DISCUSSION

During development and lineage specification, neuronal cells activate and regulate certain genetic patterns. An additional layer of this patterning derives from epigenetics and how chromatin modifications can affect transcriptional programs.

In this study we focused on how the epigenetic landscape, safeguards specific gene expression batteries and how alteration of it, can lead to cellular dysfunction and disease.

We investigated how aberrant gene silencing caused by hypermethylated gene promoters is linked with tumor formation and how targeting such feature can be crucial for better prognosis. Hitherto, *EPAS1* has been considered as an oncogene in neuroblastoma formation. Our data navigate *EPAS1* towards the opposite direction, as a tumor suppressor. Use of the already FDA approved demethylating drug AZA, results to better survival in xenografted tumors. However, since the link between DNA demethylation and HIF2a as a tumor suppressor is not clear yet, further research should be done on how surveillance of gene silencing could be a clinical target for neuroblastoma treatment.

More extensively in this piece of work, we studied postmitotic neurons of the CNS, the dopaminergic and serotonergic neurons and how histone modifications contribute to the acquisition of their identity during development and maintenance of it throughout adulthood. Initially, we implemented a protocol that, for the time being, was the only that could allow ChIP sequencing from a low number of cells. Combining the ChIPseq with RNA seq we were able to associate developmental stages with chromatin states. NPCs were differentiated to postmitotic dopaminergic or serotonergic neurons in accordance with specific chromatin patterns. Differentially expressed genes between the three different cell types primarily belonged to the K4 state indicating a major role of the transcription factors combined with other posttranslational modifications, rather than the repressive histones K27 and K9. Distribution of K27 around the TSS was depending on whether the modification was alone or combined with K4 or K9. Early developmental genes that were silenced in NPCs, acquired an additional layer of repression with K9 ensuring that silence will be maintained. The bivalent state K4/K27 was the most frequent combination containing the K27 mark in our cell types. During transition from NPC to differentiated state, bivalency was resolved to K4 accordingly. As a matter of fact, when mDA neurons got stressed pharmacologically, bivalent genes were upregulated and genes containing the additional repressive layer of K9 did not show increased expression.

To further understand how bivalency and PRC2 contribute to cell type specification and survival, we investigated the role of PRC2 further.

PRC2 has been shown to regulate neurogenesis in the neocortex[81,82] however, its role in postmitotic neurons is yet unknown. Previous studies have shown that PRC2 in MSNs and Purkinje cells is necessary for silencing of death promoting genes, responsible for neurodegeneration[83] . Ablation of H3K27me3 by targeting the methyltransferases Ezh1 and Ezh2 resulted in progressive neuronal atrophy and cell death in those neurons. That was the first study shedding light in the field, with further researchers focusing on PRC2 complex in different neuronal populations. Depletion of the methyltransferase Ezh2 from the Polycomb complex in Pitx3 expressing mDA neurons resulting in progressive loss of VTA dopaminergic neurons. However, due to methyltransferase activity of Ezh1, the levels of H3K27me3 were not affected in the mDA neurons, hence the observed cell death might have been uncoupled from the canonical PRC2 activity[84].

In our study we targeted PRC2 activity by removing EED, the component that is responsible for assembling the complex, in dopaminergic and serotonergic neurons. We observed a progressive loss of H3K27me3 accompanied by gene expression changes 8 months after removal of the modification. Upregulation of PRC2 repressed developmental genes was observed, as well as downregulation of key identity genes resulting to malfunction of those neurons. Interestingly, no cell death was noted even in later timepoints as of 16 months of age. The “cell death” promoting genes as previously described [83] were indeed upregulated, however that didn’t lead to similar outcome and cell number was not altered. Cell type specific effects or experimental approaches could be responsible for such discrepancies between studies, however it is undoubtful that PRC2 function is necessary for maintenance of identity and/or survival of postmitotic neurons. Further experimental evidence though are necessary to highlight the function of this mechanism, focusing either on the origin and characteristics of derepressed genes, transcriptional dynamics and neuronal characteristics that makes them less or more vulnerable to such perturbations.

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6 REFERENCES

1 Vaquerizas, J. M., Kummerfeld, S. K., Teichmann, S. A. & Luscombe, N. M. A census of human transcription factors: function, expression and evolution. *Nature Reviews Genetics* **10**, 252-263, doi:10.1038/nrg2538 (2009).

2 Li, B., Carey, M. & Workman, J. L. The role of chromatin during transcription. *Cell* **128**, 707-719, doi:10.1016/j.cell.2007.01.015 (2007).

3 Lewis, E. B. A gene complex controlling segmentation in Drosophila. *Nature* **276**, 565-570, doi:10.1038/276565a0 (1978).

4 Schuettengruber, B., Bourbon, H. M., Di Croce, L. & Cavalli, G. Genome Regulation by Polycomb and Trithorax: 70 Years and Counting. *Cell* **171**, 34-57, doi:10.1016/j.cell.2017.08.002 (2017).

5 Wysocka, J. *et al.* A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature* **442**, 86-90, doi:10.1038/nature04815 (2006).

6 Muramatsu, D., Kimura, H., Kotoshiba, K., Tachibana, M. & Shinkai, Y. Pericentric H3K9me3 Formation by HP1 Interaction-defective Histone Methyltransferase Suv39h1. *Cell Struct Funct* **41**, 145-152, doi:10.1247/csf.16013 (2016).

7 Margueron, R. *et al.* Role of the polycomb protein EED in the propagation of repressive histone marks. *Nature* **461**, 762-767, doi:10.1038/nature08398 (2009).

8 Højfeldt, J. W. *et al.* Accurate H3K27 methylation can be established de novo by SUZ12-directed PRC2. *Nature Structural & Molecular Biology* **25**, 225-232, doi:10.1038/s41594-018-0036-6 (2018).

9 Jung, H. R., Pasini, D., Helin, K. & Jensen, O. N. Quantitative mass spectrometry of histones H3.2 and H3.3 in Suz12-deficient mouse embryonic stem cells reveals distinct, dynamic post-translational modifications at Lys-27 and Lys-36. *Mol Cell Proteomics* **9**, 838-850, doi:10.1074/mcp.M900489-MCP200 (2010).

10 Ferrari, K. J. *et al.* Polycomb-dependent H3K27me1 and H3K27me2 regulate active transcription and enhancer fidelity. *Mol Cell* **53**, 49-62, doi:10.1016/j.molcel.2013.10.030 (2014).

11 Cao, R. & Zhang, Y. SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. *Mol Cell* **15**, 57-67, doi:10.1016/j.molcel.2004.06.020 (2004).

12 Jiao, L. & Liu, X. Structural basis of histone H3K27 trimethylation by an active polycomb repressive complex 2. *Science* **350**, aac4383, doi:10.1126/science.aac4383 (2015).

13 Montgomery, N. D. *et al.* The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation. *Curr Biol* **15**, 942-947, doi:10.1016/j.cub.2005.04.051 (2005).

14 Pasini, D., Bracken, A. P., Jensen, M. R., Lazzerini Denchi, E. & Helin, K. Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. *Embo j* **23**, 4061-4071, doi:10.1038/sj.emboj.7600402 (2004).

15 Hansen, K. H. *et al.* A model for transmission of the H3K27me3 epigenetic mark. *Nature Cell Biology* **10**, 1291-1300, doi:10.1038/ncb1787 (2008).

16 O'Carroll, D. *et al.* The polycomb-group gene Ezh2 is required for early mouse development. *Mol Cell Biol* **21**, 4330-4336, doi:10.1128/mcb.21.13.4330-4336.2001 (2001).

17 Meshorer, E. *et al.* Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. *Dev Cell* **10**, 105-116, doi:10.1016/j.devcel.2005.10.017 (2006).

18 Efroni, S. *et al.* Global transcription in pluripotent embryonic stem cells. *Cell Stem Cell* **2**, 437-447, doi:10.1016/j.stem.2008.03.021 (2008).

19 Gaspar-Maia, A. *et al.* Chd1 regulates open chromatin and pluripotency of embryonic stem cells. *Nature* **460**, 863-868, doi:10.1038/nature08212 (2009).

20 Bernstein, B. E. *et al.* A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**, 315-326, doi:10.1016/j.cell.2006.02.041 (2006).

21 Azuara, V. *et al.* Chromatin signatures of pluripotent cell lines. *Nat Cell Biol* **8**, 532-538, doi:10.1038/ncb1403 (2006).

22 Meissner, A. *et al.* Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* **454**, 766-770, doi:10.1038/nature07107 (2008).

23 Fouse, S. D. *et al.* Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation. *Cell Stem Cell* **2**, 160-169, doi:10.1016/j.stem.2007.12.011 (2008).

24 Liu, J., Wu, X., Zhang, H., Pfeifer, G. P. & Lu, Q. Dynamics of RNA Polymerase II Pausing and Bivalent Histone H3 Methylation during Neuronal Differentiation in Brain Development. *Cell Rep* **20**, 1307-1318, doi:10.1016/j.celrep.2017.07.046 (2017).

25 Gargiulo, G. *et al.* NA-Seq: A Discovery Tool for the Analysis of Chromatin Structure and Dynamics during Differentiation. *Developmental Cell* **16**, 466-481, doi:https://doi.org/10.1016/j.devcel.2009.02.002 (2009).

26 Mikkelsen, T. S. *et al.* Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* **448**, 553-560, doi:10.1038/nature06008 (2007).

27 Cui, K. *et al.* Chromatin signatures in multipotent human hematopoietic stem cells indicate the fate of bivalent genes during differentiation. *Cell Stem Cell* **4**, 80-93, doi:10.1016/j.stem.2008.11.011 (2009).

28 Hawkins, R. D. *et al.* Distinct epigenomic landscapes of pluripotent and lineage-committed human cells. *Cell Stem Cell* **6**, 479-491, doi:10.1016/j.stem.2010.03.018 (2010).

29 Agger, K. *et al.* UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature* **449**, 731-734, doi:10.1038/nature06145 (2007).

30 Smallwood, A., Estève, P. O., Pradhan, S. & Carey, M. Functional cooperation between HP1 and DNMT1 mediates gene silencing. *Genes Dev* **21**, 1169-1178, doi:10.1101/gad.1536807 (2007).

31 Roeper, J. Dissecting the diversity of midbrain dopamine neurons. *Trends Neurosci* **36**, 336-342, doi:10.1016/j.tins.2013.03.003 (2013).

32 Ye, W., Shimamura, K., Rubenstein, J. L., Hynes, M. A. & Rosenthal, A. FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755-766, doi:10.1016/s0092-8674(00)81437-3 (1998).

33 Ono, Y. *et al.* Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. *Development* **134**, 3213-3225, doi:10.1242/dev.02879 (2007).

34 Alavian, K. N., Scholz, C. & Simon, H. H. Transcriptional regulation of mesencephalic dopaminergic neurons: the full circle of life and death. *Mov Disord* **23**, 319-328, doi:10.1002/mds.21640 (2008).

35 Chakrabarty, K. *et al.* Genome wide expression profiling of the mesodiencephalic region identifies novel factors involved in early and late dopaminergic development. *Biol Open* **1**, 693-704, doi:10.1242/bio.20121230 (2012).

36 Puelles, E. *et al.* Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. *Development* **131**, 2037-2048, doi:10.1242/dev.01107 (2004).

37 Smidt, M. P. *et al.* A homeodomain gene Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons. *Proc Natl Acad Sci U S A* **94**, 13305-13310, doi:10.1073/pnas.94.24.13305 (1997).

38 Smidt, M. P. *et al.* A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. *Nat Neurosci* **3**, 337-341, doi:10.1038/73902 (2000).

39 Vernay, B. *et al.* Otx2 regulates subtype specification and neurogenesis in the midbrain. *J Neurosci* **25**, 4856-4867, doi:10.1523/jneurosci.5158-04.2005 (2005).

40 Saucedo-Cardenas, O. *et al.* Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc Natl Acad Sci U S A* **95**, 4013-4018, doi:10.1073/pnas.95.7.4013 (1998).

41 Andersson, E., Jensen, J. B., Parmar, M., Guillemot, F. & Björklund, A. Development of the mesencephalic dopaminergic neuron system is compromised in the absence of neurogenin 2. *Development* **133**, 507-516, doi:10.1242/dev.02224 (2006).

42 Kele, J. *et al.* Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. *Development* **133**, 495-505, doi:10.1242/dev.02223 (2006).

43 Smits, S. M., Ponnio, T., Conneely, O. M., Burbach, J. P. & Smidt, M. P. Involvement of Nurr1 in specifying the neurotransmitter identity of ventral midbrain dopaminergic neurons. *Eur J Neurosci* **18**, 1731-1738, doi:10.1046/j.1460-9568.2003.02885.x (2003).

44 Wallén, A. A. *et al.* Orphan nuclear receptor Nurr1 is essential for Ret expression in midbrain dopamine neurons and in the brain stem. *Mol Cell Neurosci* **18**, 649-663, doi:10.1006/mcne.2001.1057 (2001).

45 Zetterström, R. H. *et al.* Dopamine neuron agenesis in Nurr1-deficient mice. *Science* **276**, 248-250, doi:10.1126/science.276.5310.248 (1997).

46 Qi, Z., Miller, G. W. & Voit, E. O. in *Systems Biology of Parkinson's Disease* (eds Peter Wellstead & Mathieu Cloutier) 151-171 (Springer New York, 2012).

47 Barnes, N. M. & Sharp, T. A review of central 5-HT receptors and their function. *Neuropharmacology* **38**, 1083-1152, doi:10.1016/s0028-3908(99)00010-6 (1999).

48 Hannon, J. & Hoyer, D. Molecular biology of 5-HT receptors. *Behav Brain Res* **195**, 198-213, doi:10.1016/j.bbr.2008.03.020 (2008).

49 Gaspar, P., Cases, O. & Maroteaux, L. The developmental role of serotonin: news from mouse molecular genetics. *Nat Rev Neurosci* **4**, 1002-1012, doi:10.1038/nrn1256 (2003).

50 Erickson, J. T., Shafer, G., Rossetti, M. D., Wilson, C. G. & Deneris, E. S. Arrest of 5HT neuron differentiation delays respiratory maturation and impairs neonatal homeostatic responses to environmental challenges. *Respir Physiol Neurobiol* **159**, 85-101, doi:10.1016/j.resp.2007.06.002 (2007).

51 Fernandez, S. P. & Gaspar, P. Investigating anxiety and depressive-like phenotypes in genetic mouse models of serotonin depletion. *Neuropharmacology* **62**, 144-154, doi:10.1016/j.neuropharm.2011.08.049 (2012).

52 Eley, T. C. *et al.* Gene–environment interaction analysis of serotonin system markers with adolescent depression. *Molecular Psychiatry* **9**, 908-915, doi:10.1038/sj.mp.4001546 (2004).

53 Fernandez, S. P. *et al.* Constitutive and Acquired Serotonin Deficiency Alters Memory and Hippocampal Synaptic Plasticity. *Neuropsychopharmacology* **42**, 512-523, doi:10.1038/npp.2016.134 (2017).

54 Li, Y. *et al.* Serotonin neurons in the dorsal raphe nucleus encode reward signals. *Nature Communications* **7**, 10503, doi:10.1038/ncomms10503 (2016).

55 Veenstra-VanderWeele, J. *et al.* Autism gene variant causes hyperserotonemia, serotonin receptor hypersensitivity, social impairment and repetitive behavior. *Proc Natl Acad Sci U S A* **109**, 5469-5474, doi:10.1073/pnas.1112345109 (2012).

56 Lerch-Haner, J. K., Frierson, D., Crawford, L. K., Beck, S. G. & Deneris, E. S. Serotonergic transcriptional programming determines maternal behavior and offspring survival. *Nature Neuroscience* **11**, 1001-1003, doi:10.1038/nn.2176 (2008).

57 Steinbusch, H. W. Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. *Neuroscience* **6**, 557-618, doi:10.1016/0306-4522(81)90146-9 (1981).

58 Gaspar, P. & Lillesaar, C. Probing the diversity of serotonin neurons. *Philos Trans R Soc Lond B Biol Sci* **367**, 2382-2394, doi:10.1098/rstb.2011.0378 (2012).

59 Spencer, W. C. & Deneris, E. S. Regulatory Mechanisms Controlling Maturation of Serotonin Neuron Identity and Function. *Front Cell Neurosci* **11**, 215, doi:10.3389/fncel.2017.00215 (2017).

60 Rood, B. D. *et al.* Dorsal raphe serotonin neurons in mice: immature hyperexcitability transitions to adult state during first three postnatal weeks suggesting sensitive period for environmental perturbation. *J Neurosci* **34**, 4809-4821, doi:10.1523/jneurosci.1498-13.2014 (2014).

61 Okaty, B. W. *et al.* Multi-Scale Molecular Deconstruction of the Serotonin Neuron System. *Neuron* **88**, 774-791, doi:10.1016/j.neuron.2015.10.007 (2015).

62 Fishell, G. & Heintz, N. The neuron identity problem: form meets function. *Neuron* **80**, 602-612, doi:10.1016/j.neuron.2013.10.035 (2013).

63 Hobert, O., Carrera, I. & Stefanakis, N. The molecular and gene regulatory signature of a neuron. *Trends Neurosci* **33**, 435-445, doi:10.1016/j.tins.2010.05.006 (2010).

64 Kadkhodaei, B. *et al.* Transcription factor Nurr1 maintains fiber integrity and nuclear-encoded mitochondrial gene expression in dopamine neurons. *Proc Natl Acad Sci U S A* **110**, 2360-2365, doi:10.1073/pnas.1221077110 (2013).

65 Kadkhodaei, B. *et al.* Nurr1 is required for maintenance of maturing and adult midbrain dopamine neurons. *J Neurosci* **29**, 15923-15932, doi:10.1523/jneurosci.3910-09.2009 (2009).

66 Schmidt, M. *et al.* The bHLH transcription factor Hand2 is essential for the maintenance of noradrenergic properties in differentiated sympathetic neurons. *Dev Biol* **329**, 191-200, doi:10.1016/j.ydbio.2009.02.020 (2009).

67 Zhao, Z. Q. *et al.* Lmx1b is required for maintenance of central serotonergic neurons and mice lacking central serotonergic system exhibit normal locomotor activity. *J Neurosci* **26**, 12781-12788, doi:10.1523/jneurosci.4143-06.2006 (2006).

68 Coppola, E., d'Autréaux, F., Rijli, F. M. & Brunet, J. F. Ongoing roles of Phox2 homeodomain transcription factors during neuronal differentiation. *Development* **137**, 4211-4220, doi:10.1242/dev.056747 (2010).

69 Stott, S. R. *et al.* Foxa1 and foxa2 are required for the maintenance of dopaminergic properties in ventral midbrain neurons at late embryonic stages. *J Neurosci* **33**, 8022-8034, doi:10.1523/jneurosci.4774-12.2013 (2013).

70 Liu, C. *et al.* Pet-1 is required across different stages of life to regulate serotonergic function. *Nature Neuroscience* **13**, 1190-1198, doi:10.1038/nn.2623 (2010).

71 Liu, A. & Joyner, A. L. Early anterior/posterior patterning of the midbrain and cerebellum. *Annu Rev Neurosci* **24**, 869-896, doi:10.1146/annurev.neuro.24.1.869 (2001).

72 Fox, S. R. & Deneris, E. S. Engrailed Is Required in Maturing Serotonin Neurons to Regulate the Cytoarchitecture and Survival of the Dorsal Raphe Nucleus. *The Journal of Neuroscience* **32**, 7832, doi:10.1523/JNEUROSCI.5829-11.2012 (2012).

73 Shilatifard, A. Molecular implementation and physiological roles for histone H3 lysine 4 (H3K4) methylation. *Curr Opin Cell Biol* **20**, 341-348, doi:10.1016/j.ceb.2008.03.019 (2008).

74 O'Meara, M. M., Zhang, F. & Hobert, O. Maintenance of neuronal laterality in Caenorhabditis elegans through MYST histone acetyltransferase complex components LSY-12, LSY-13 and LIN-49. *Genetics* **186**, 1497-1502, doi:10.1534/genetics.110.123661 (2010).

75 Pasini, D. & Di Croce, L. Emerging roles for Polycomb proteins in cancer. *Curr Opin Genet Dev* **36**, 50-58, doi:10.1016/j.gde.2016.03.013 (2016).

76 Hoebeeck, J. *et al.* Aberrant methylation of candidate tumor suppressor genes in neuroblastoma. *Cancer Lett* **273**, 336-346, doi:10.1016/j.canlet.2008.08.019 (2009).

77 Kiss, N. B. *et al.* Quantitative global and gene-specific promoter methylation in relation to biological properties of neuroblastomas. *BMC Medical Genetics* **13**, 83, doi:10.1186/1471-2350-13-83 (2012).

78 Christman, J. K. 5-Azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* **21**, 5483-5495, doi:10.1038/sj.onc.1205699 (2002).

79 Coffey, D. C. *et al.* The histone deacetylase inhibitor, CBHA, inhibits growth of human neuroblastoma xenografts in vivo, alone and synergistically with all-trans retinoic acid. *Cancer Res* **61**, 3591-3594 (2001).

80 Esteller, M. Epigenetics in Cancer. *New England Journal of Medicine* **358**, 1148-1159, doi:10.1056/NEJMra072067 (2008).

81 Hirabayashi, Y. *et al.* Polycomb limits the neurogenic competence of neural precursor cells to promote astrogenic fate transition. *Neuron* **63**, 600-613, doi:10.1016/j.neuron.2009.08.021 (2009).

82 Tsuboi, M., Hirabayashi, Y. & Gotoh, Y. Diverse gene regulatory mechanisms mediated by Polycomb group proteins during neural development. *Curr Opin Neurobiol* **59**, 164-173, doi:10.1016/j.conb.2019.07.003 (2019).

83 von Schimmelmann, M. *et al.* Polycomb repressive complex 2 (PRC2) silences genes responsible for neurodegeneration. *Nat Neurosci* **19**, 1321-1330, doi:10.1038/nn.4360 (2016).

84 Wever, I., von Oerthel, L., Wagemans, C. & Smidt, M. P. EZH2 Influences mdDA Neuronal Differentiation, Maintenance and Survival. *Front Mol Neurosci* **11**, 491, doi:10.3389/fnmol.2018.00491 (2018).